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EUROPEAN PATENT APPLICATION

21 Application number: 84810600.1

61 Int. Cl.⁴: **G 01 N 21/75, G 01 N 33/543,**
G 01 N 21/64, G 01 N 33/72

22 Date of filing: 10.12.84

43 Date of publication of application: 18.06.86
Bulletin 86/25

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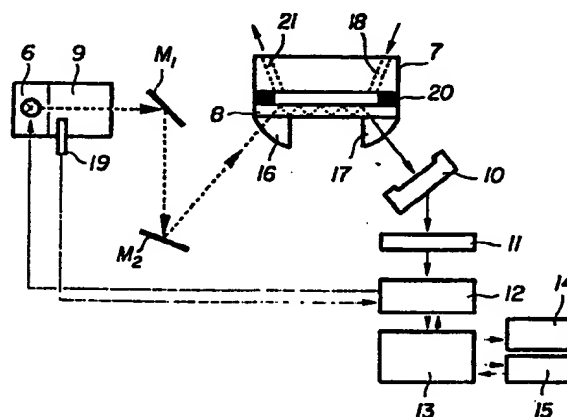
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84 Designated Contracting States: **AT BE CH DE FR GB IT LI LU NL SE**

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54 **Method for optically ascertaining parameters of species in a liquid analyte.**

57 An optical waveguide with one or more coatings of reactants specific to species to be analyzed in a liquid analyte immersed therein. A light signal carried by the waveguide undergoes interaction either with the bulk analyte whereby a first signal in connection with the bulk analyte is obtained and, simultaneously, with a layer of complex resulting from the reaction of one of said specific reactant with one of said species or with two or more complex layers corresponding to two or more of said species, this generating a second (or more) signal to provide the required analytical information on said species.



METHOD FOR OPTICALLY ASCERTAINING PARAMETERS OF

SPECIES IN A LIQUID ANALYTE

The present invention concerns a method for ascertaining parameters in a liquid analyte, e.g. for determining species dissolved therein.

This method relates to the known techniques in which an optical
5 waveguide carrying a totally reflected light signal is contacted with
an analyte and the evanescent wave component of said signal interacts with the analyte at the solid-liquid interface in a manner responsive to some parameters inherent to the species therein. For instance, information on such parameters may concern an absorption of
10 part of the luminous energy of the incident signal at the points of reflection thereof within the waveguide by the molecules of the species of interest at said interface or to an excitation of some fluorophores by said luminous energy with the consecutive production of a fluorescence signal characteristic of such species. In general,
15 the interaction is limited to the region corresponding to the depth of penetration into the analyte of the evanescent wave component of the incident light, this depth ranging from a few angströms to some hundredths of angströms starting from the waveguide surface.

Although it is generally known that the aforementioned interaction
20 may provide information on some parameters in the bulk solution (Hardy, USP 4,050,895), recently published work has shown that improved results (i.e. better sensitivity and accuracy) are obtained when the effective interaction of the evanescent wave component with the analyte (or rather, the leaking into the analyte of a substantial
25 portion of the energy thereof) is limited to involve a single layer (generally monomolecular) of compounds of interest bound to the surface of the waveguide. In other words, one has recently found that a very useful new analytical technique can be based on attaching to the waveguide, before contacting it with the analyte to be
30 investigated, a reactant specific to a species of said analyte and, thereafter, immersing it into said analyte; in these conditions, the species of interest will bind to the said reactant and provide at

the surface of the waveguide a layer of a complex whose content of the species of interest (i.e. the actual density of said species in the area of interaction) will grow very rapidly with time and provide increased interaction with the light travelling in the waveguide and stronger response at the output thereof (KRONICK and LITTLE, USP 3,939,350).

In such types of analysis involving the formation of a very thin layer of product of interest on the waveguide surface, the interaction of the light signal with the bulk of the solution is considered a nuisance (background noise) and attempts have been made to minimize it as much as possible. For instance, compromising condition between maximal interaction of the evanescent component with the monomolecular layer deposited on the waveguide surface and minimal interaction with the bulk solution can be achieved by controlling the depth of penetration of said evanescent component outside the material of the waveguide at the waveguide/analyte interface. Such control can be effected by selecting a waveguide with a suitable refractive index n_1 relative to that (n_2) of the solution and by choosing adequately the angle of total reflection in the guide as well as the wavelength of the incident light (more detailed explanation on why this can be so and how to make such selections will be provided hereafter). For instance in co-pending application EP-A-75353, it has been disclosed that this depth of penetration can be optimized to match with the thickness of said layer of interest or to be in excess thereof.

However, it has now been found that unexpectedly this approach is not always the most desirable; indeed, it has been found with surprise that, under some conditions, the penetration of the evanescent wave component distinctively beyond the distance corresponding to the thickness of the layer of interest can be extremely useful in concurrently providing analytical results on parameters of the analyte other than that specifically involved in said layer of interest, e.g. on species in solution in the analyte. Therefore, this discovery led to the definition of one aspect of a new analytical method outlined in claim 1 given in annexe to this specification. Moreover, this method was also extended to further aspects after realizing that useful interactions of the light carried by the waveguide with the analyte may involve more than on specific area of

the waveguide (i.e. the area where interaction takes place with either a single layer coating on this area or with the bulk solution or, simultaneously with both bulk and coating), i.e. may involve two or several distinctly different areas of said waveguide. For instance,
5 in one area the interaction will take place with the bulk solution and in another or more optically separated areas the interaction will take place with one or several layers of interest. Annexed claim 1 actually summarizes the complete scope of this method of the invention.

10 In the method of the invention, any type of interaction of the light contained in the waveguide with the analyte can be considered. Thus, this interaction can result from absorption of part of the signal, the output response being then a decrease in output energy gathered by the collecting and detecting means located at the out-
15 let of the waveguide. Alternatively, the interaction may result in the production of fluorescence if the species of interest (whether located in the bulk analyte or in a coating of interest at the waveguide surface) can generate fluorescence under excitation by the incident signal. Such is the case for instance in fluorescence type as-
20 says where one of the partners in a complex under formation on the surface of the waveguide comprises a fluorophore group which induces fluorescence upon formation of said complex. Otherwise, useful responses can also result from the scattering of the incident light by molecular aggregates built on the surface of the illuminated wave-
25 guide.

In order to practically carry out the method of the invention, one can for instance use a waveguide in the form of an optical fiber or a glass or plastic slide coated with a reagent specific to one first species dissolved in an analyte which additionally contains
30 another or more species of interest. In an example to be developed in detail hereinafter, this first species can be a specific hemoglobin compound in a sample of blood also containing other hemoglobin or blood factors. Thus, in this case, the waveguide will carry, attached thereto, an antibody specific to that first species and, upon
35 contacting the lit waveguide with the blood sample and arranging the measurement conditions (as specified hereafter) for providing an effective and signal generating interaction of the light in the wave-

guide with, simultaneously, the bulk solution and a monomolecular layer of a complex involving the antibody and that first species that forms on the waveguide surface, a signal is provided at the outlet of the guide, this signal being representative concurrently or simultaneously of the total hemoglobin (or other blood factors) having interacted in bulk with the excitation signal and of said first species involved in the formation of the complex.

In this case, the signal at the outlet of the waveguide is representative of two independent effects and can be decoded by simple means since the response to the bulk hemoglobin corresponds to an instantaneous partial extinction of the signal output collected from the waveguide end (this is actually the aforementioned background noise) while the response to the thin layer is a time dependent signal due to the formation of said complex layer of antibody and the first specific species to be determined which is a rate reaction.

Otherwise, if two specific factors in the analyte (for instance factor 1 and factor 2 among others present) must be determined, a waveguide with two independently working optical areas are preferably selected, each area being provided with one reagent (antibody) specific to one of said factors to be determined. In such case, the two response signals collected at the output of the waveguide (this being so if the waveguide is not provided already with separate outputs) are either (a) phase dependent or (b) frequency dependent.

Case (a) may be illustrated by a waveguide provided with two independent optical elements such as the two oppositely facing parallel walls of an analytical cuvette, said walls being light conductive for a fully reflected signal and each internally coated with one of the two aforementioned reactants, each being specific to one of the two factors (factor 1 and factor 2) to be determined in the analyte. In this case the two elements are illuminated in turn (alternatively applied pulses), the mode of application being also used for synchronization purposes at the detecting and processing end to ensure proper separation and independent display of the corresponding signals.

Case (b) may be illustrated by a waveguide structure comprising two physically separated areas on the same light path of the waveguide (i.e. not separated optically) but providing responses at two

different wavelengths (this can be implemented, for instance, by having one area responsive to absorption, e.g. at the excitation wavelength and another area providing a fluorescent response, i.e. a signal of a wavelength different from the excitation wavelength). In

5 this case the detector unit is provided with means to separate the components of the output consisting of a signal with two wavelengths into individual signals by usual means (band-pass filters or dichroic beam splitters). Such a situation can result for instance from attaching to a first area of the waveguide a first reagent specific
10 of factor No.1 to be analyzed, the layer of reaction product being light absorbing, and attaching to a second area of the waveguide a second reagent specific of factor No.2, the reaction product of said second reagent and factor No.2 being fluorescent under excitation by the incident light.

15 Of course case (b) can also be illustrated by a variant of the structure of case (a), i.e. with one of the separately illuminated waveguide element being responsive to absorption while the other is fluorescence responsive.

The practical aspect of the invention will now be illustrated
20 with reference to actual analysis cases. The first case refers to blood analysis and, more particularly, to the direct determination in blood samples of hemoglobin and various other hemoglobin factors such as glycosylated hemoglobin, this being, if desired, relative to total hemoglobin in this sample.

25 Glycosylated hemoglobins (HbA_{1a} , A_{1b} and A_{1c}) are important factors in the diagnosis and monitoring of patients with diabetes. The determination of the content of HbA_{1c} (which amounts to about 80% of total glycosylated hemoglobins (HbA_1)) relative to total hemoglobin (i.e. HbA_0 , non-glycosylated hemoglobin plus (HbA_1)) is particularly important with regard to that disease.
30

Hemoglobin A_{1c} is a glycohemoglobin with an amino acid structure which is identical to that of HbA_0 ; the important difference is the presence of 1-amino-1-deoxy-fructose attached in the 2,3-diphosphoglycerate pocket to the N-terminal valine in the beta-chain
35 of HbA_{1c} . The modification of HbA_0 to HbA_{1c} is a continuous non-enzymatic post-translational process, the rate of which is a function of the blood glucose concentration. Glycosylation occurs as a two

step process. Firstly, the open aldehydic form of glucose reacts with the terminal amino group of the beta-chains of Hb to form a SCHIFF base. Secondly, the SCHIFF base then undergoes an AMADORI rearrangement to form HbA_{1c}. The intermediate SCHIFF base is unstable with a 60-fold greater tendency to dissociate (to the free sugar plus protein) than the stable ketoamine of HbA_{1c}. As only a small fraction of blood glucose is in the open aldehydic form (approximately 0.001%) and the rate of ketoamine formation is slow (although effectively irreversible), the formation of HbA_{1c} is an indication of long term blood glucose concentration. Over the 120 days life-span of the human red blood cell, the number of glycosylated Hb molecules increases proportionally to the mean blood glucose concentration. The relationship between the mean plasma glucose and HbA_{1c} concentrations is unique in that a single HbA_{1c} measurement provides a retrospective assessment of blood glucose control over the preceeding 6 to 8 weeks. It is generally accepted that HbA_{1c} measurements are a very useful tool in monitoring diseases of carbohydrate metabolism, specifically diabetes mellitus. Diabetics have high long-term blood sugar levels and this is reflected in their HbA_{1c} levels. Normal adults have about 3-6% of their total hemoglobin as HbA_{1c} whereas the range in juvenile and maturity onset diabetics is 6-15% as HbA_{1c}. A similar increase in HbA_{1c} concentration has been noted in mice with genetic and chemically induced diabetes and in pancreatectomized dogs.

Among the several methods which exist for determining glycosylated Hb in blood, HbA₁ and in particular HbA_{1c} measurements have now become a method of choice for monitoring the treatment of diabetics (L. JOVANOVIĆ et al., American J. of Medicine (1981) 70, 331; D.E. GOLDSTEIN et al., Diabetes (1982) 31, 70; K.H. GABBOY et al., J. of Clinical Endocrinology and Metabolism (1977) 44, 859; B. GONEN et al., Diabetologia (1978) 15, 1; C.M. PETERSON, Diabetes (1982) 31, 1). Also, the following patent documents can be usefully mentioned: US-A-4.247.553; GB-A-1.580.318; US-A-4.222.836; US-4.372.747; 4.200.435; 4.341.635. These methods can be readily classified by the mechanism used to separate glycosylated Hb from non-glycosylated Hb. For instance ion-exchange chromatography was used early and is still the most common method (H.G. KUNKEL et al., Science (1955) 122, 288). Although such ion-exchange technique is currently the only available

method which specifically measures HbA_{1c}, it has a number of limitations of which temperature and pH sensitivity are the most important. Ion-exchange also is subject to interferences as labile glycosylated Hb (pre-HbA_{1c}) must be removed prior to assay and both fetal Hb (HbF) and Sick Cell Hb (HbS) interfere with the results.

Other techniques involve agar gel electrophoresis (L. MENARD et al., Clinical Chemistry (1980) 26, 1598), isoelectric focusing (K.M. SPICER et al., Diabetes (1978) 27, 384), colorimetry, e.g. with thiobarbituric acid (R. FLUCKIGER et al., FEBS Letters (1976) 71, 356) and affinity chromatography (V. BOURIOTIS et al., Diabetologia (1981) 21, 579). Only one type radioimmunoassay has been reported (J. JAVID et al., British J. of Haematology (1978) 38, 329) which was slow (more than 3 days to work) and technically complex as requiring the preparations of radiolabelled HbA_{1c}. Although the methods of the prior art have merits, there is still a need for methods providing quick results (less than about 15 minutes), requiring less skilled operators and less costly to be undertaken on a routine basis. Current art methods are slow (typically more than 1 hour results), technically complicated (require more than five pipetting manipulation steps) and unsuited to testing outside a laboratory environment. Further, present methods require that total hemoglobin be ascertained separately from the glycosylated factors and it would be desirable that both analytical data can be ascertained substantially together and correlated without delay.

The method of the present invention, as summarized in claim 9, remedies the inconvenience of prior art methods and further offers the advantage of directly relating, if desired, the percent of glycosylated factor or other hemoglobin factors to total hemoglobin.

The present method allows for the separate determination of (Hb) A_{1c}, A_{1a} or A_{1b} provided antibodies specific to any of such species are available in purified form. Otherwise, using less specific antibodies, the present method allows for the combined determination of two or more blood factors taken together, i.e. all glycosylated Hb relative to total Hb for instance. Of course, the method also provides for the determination of blood factors other than the ones hereabove if corresponding reagents specific to said factors in complex formation reactions are available (e.g. HbF, HbS or other human hemo-

globin variants.

The present invention does not concern the obtention or preparation of such specifically reactive complex moieties (monoclonal or polyclonal antibodies) which belong to the prior art but it concerns their use as coating materials in the preparation of the active wave guides to be contacted with the blood sample to be analyzed according to the invention.

The waveguides used in the present methods can be of many kinds and some have been disclosed in copending application EP-A-75353 together with the methods for coating the wave guides with the reactive antibodies selected.

In the present case, plate-like or fiber optic waveguides included as members of an analytical cuvette are preferably used, the coated surface of the wave guides being put into contact with the blood sample once the latter has been injected into the cuvette.

The optical technique used here relates, as discussed hereinabove, mainly to light absorption, i.e. there is an interaction of the evanescent component of the wave transported in the guide with the molecules, first in the ambient liquid (the depth of penetration of the evanescent component exceeds somewhat the thickness of the antibody coating, which provides an instantaneous response) and, second, with the Hb-antibody complex which starts building up on the guide in forms of an additional layer due to the reaction of the blood factor to be determined with the specific complex moiety (antibody) previously coated on the guide surface. Although the depth of interaction of the evanescent light component is not substantially limited to the thickness of the layer of the complex, it has been surprisingly found that the optical response to that build-up is independent of the bulk absorption due to the blood itself and the two effects can be easily distinguished without sophisticated techniques for decoding the signals originating from one or the other effect.

Hb derivatives have characteristic absorption spectra dependent on their chemical state. Hence, any of the usual absorptiometric techniques are equally applicable for implementing the invention (L. TEN-TORI et al., Hemoglobin, in Methods in Enzymology (1981), vol. 76, 707-732, Academic Press, New-York). Included are the cyanomethemoglobin method and single or multi-wavelength absorptionmetric assays,

preferably in the rang 400 to 600 nm, specifically 400-420 nm and 550-600 nm. Also included are such isobestic point methods where the absorption by the Hb molecule is independent of the degree of oxygen saturation.

5 The invention and its present illustrative aspect will be better understood with help of the accompanying drawing.

Fig. 1a is a diagram explaining the propagation of totally reflected light in a medium (wave guide) of refractive index n_1 greater than n_2 , the refractive index of another medium (analyte) with which
10 the wave guide is in contact.

Fig. 1b is appendent to Fig. 1a and schematically represents the penetration of the evanescent wave component in the rarer medium (analyte).

Fig. 2 is a schematic layout of a single waveguide apparatus
15 for carrying out the method of the invention.

Fig. 3 is a schematic layout of another embodiment of an apparatus for carrying out the method of the invention.

Fig. 4a is a schematic top view of a detail of another embodiment of an analytical apparatus involving a double waveguide cell.

20 Fig. 4b is a schematic view of a variant of the embodiment of fig. 4a.

Fig. 4c is a schematic view of still another embodiment.

Fig. 5 is a schematized representation of the phenomena occurring during an analysis according to the method of the invention.

25 Fig. 6 is a diagram showing response curves in performing an analysis according to one embodiment of the invention.

Fig. 7 is a diagram showing the variation of one parameter of the response curve as a function of the angle of incidence of the multiply reflected light beam travelling through the waveguide.

30 Fig. 8 is a diagram with a typical response curve in the analysis of $\text{HbA}_{1\text{C}}$ in the presence of hemoglobin.

Fig. 9 illustrates another type of analysis involving fluorescence.

As said before, when a light beam 1 strikes with an angle the
35 interface between two transparent media n_1 and n_2 (fig. 1a), striking from the medium n_1 with the greater refractive index ($n_1 > n_2$), total internal reflection occurs (N.J. HARRICK, Internal Reflexion

Spectroscopy, Wiley Interscience, New York (1967)) when the angle of reflection θ is larger than a certain value θ_c called the critical angle given by the equation:

$$\theta_c = \sin^{-1}(n_2/n_1) \quad 1$$

The reflected beam is indicated by numeral 2. In this case the evanescent wave penetrates a distance (d_p) of the order of a fraction of a wavelength beyond the reflecting surface into the rarer medium of refractive index n_2 . According to Maxwell's equations a standing sinusoidal wave, perpendicular to the reflecting surface, is established in the denser medium (fig. 1b). Although there is no net energy flow into the non absorbing, rarer medium, there is an evanescent, nonpropagating field 3 in that medium, the electric field amplitude (E) of which is largest at the surface interface (E_0) and decays exponentially with distance (Z) from the surface according to:

$$E = E_0 \cdot \exp (-Z/d_p) \quad 2$$

The depth of penetration (d_p), defined as the distance required for the electric field amplitude to fall to $\exp (-1)$ of its value at the surface, is given by:

$$d_p = \frac{\lambda/n_1}{2\pi \left(\sin^2 \theta - (n_2/n_1)^2 \right)^{1/2}} \quad 3$$

Starting from 90° , as θ approaches θ_c , d_p becomes infinitely large, and at a fixed angle, increases with closer index matching (i.e., as $n_2/n_1 \rightarrow 1$). Also, because d_p is proportional to wavelength, it is greater at longer wavelengths.

Thus, by an appropriate choice of the refractive index n_1 of the transparent wave guide, of the incident angle, and of the wavelength, one can select a d_p to control optical interaction, either mainly with substances 4 close or at given distance from the interface and minimally with substances 5 beyond said distance or, with varying response ratios, with both 4 and 5. And this is precisely

one of the key factors of the present invention, i.e. to have established that appropriate selection of said parameters (n_1 , θ and λ) provides optimal conditions for measuring, at the same time, two independent parameters in the analyte. In the present embodiment, the denser medium can be constituted by a quartz microscope slide ($n_1 = 1.54$) and the rarer medium is aqueous blood sample ($n_2 = 1.34$) and θ is controllably variable so that when λ is a selected visible wavelength, d_p can be varied from about 20 to 300 nm until optimal response is provided. Of course, other materials for the waveguide can be used with refractive indices other than 1.54.

Although single reflection systems can be used, one can enhance sensitivity (lower the limit of detection) by combining the evanescent wave principle with multiple internal reflections. The number of reflections (N) is a function of the length (L) and thickness (T) of wave guide and angle of incidence (θ):

$$N = L/T \cdot \cot \theta$$

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The microscope slide waveguides used in some of the experiments had an active length of 36 mm, thickness of 1 mm and the angle of incidence varied from about 60 to 75°. Thus the number of reflections on one side for a discrete light beam was approximately 6. Similarly, in another embodiment using a fibre optic waveguide, the latter had an active length of 64 mm, a thickness of 0.6 mm and, with the same angles of incidence the total number of reflections for a discrete light beam varied from about 30 to 40.

As said before, the method of the invention can also rely on fluorescence effects. Fluorescence emission generated at a waveguide liquid interface can also be monitored at the waveguide output. As predicted by the theory of reciprocity and demonstrated with dye molecules in both monomolecular layers (CARNAGLIA and MANDEL, J. Optical Soc. of America 63, 479 (1972)) and in monodispersed spheres (LEE et al, Applied Optics 18, 862 (1979)), fluorescence emission at a waveguide/liquid interface can be treated as an evanescent wave. In effect, excitation of fluorescence by an evanescent wave produces fluorescence emission with the characteristics of an evanescent wave and thus generates an internally reflected beam of fluorescent light.

The direction of fluorescence emission of this form is mainly a function of the respective refractive index ratio and has the major characteristic (see the above CARNAGLIA reference) that photon emission has a distribution of "preferred" angular probability close to the critical angle (θ_c).

In practical terms this means that fluorescence can be monitored at the output of the waveguide in the same optical plane as the excitation light. Theoretically, this has the advantages of concentrating the fluorescent emission intensity within a small angle; also, these fluorescent photons do not pass through the bulk of the solution and thus are not subject to major optical interference (e.g. absorption, scattering).

This technique is disclosed in more detail in co-pending application EP-A-75353,

For the fluorescence measurements illustrating the present invention, the excitation wavelength was selected at 490 nm and fluorescence emission measured (wavelength greater than 510 nm) at the waveguide output by positioning cut-off filters before the photodetector element (KV 8.5; 50% transmittance at 550 nm; SCHOTT GLASS WORKS, Mainz, Germany).

Fluorescence techniques allowing for the simultaneous determination of two or more parameters, for instance with multi-analyte waveguide systems have many applications in the field of clinical diagnostics, e.g. simultaneous measurement of the thyroid hormones T_4 and T_3 , the gonadotrophins LH and FSH, tumor markers such as AFP and CEA; also the whole gamut of cell surface antigen determination as applied to clinical microbiology.

One embodiment of the apparatus used is schematically represented on fig. 2 which shows as a block diagram the major components; these components comprise a monochromator 9, a light source 6, a flow cell 7 with waveguide 8, and electronics with data-acquisition and processing microcomputer including a photomultiplier detector 10, a pre-amplifier 11, a computerized light source control system 12, a micro-processor 13, a printer 14, and a memory (floppy disc) 15.

The light source 6 in this instance was a xenon flash lamp (E.G. & G., Salem, MA) and the monochromator was equipped with a concave holographic grating (Jobin-Yvon, Paris, France) to permit a resolu-

tion of 5 nm. The flash lamp operation was controlled by microcomputer 12. To inject the samples through an input 18 to the cell 7 a programmable automatic pipette (Microlab-P; Hamilton Bonaduz AG, Bonaduz, Switzerland) was preferably used. The optical component

5 further included two mirrors M_1 and M_2 and two prisms 16 and 17. A photomultiplier tube of the detector 10 (R928; Hamamatsu, Tokyo, Japan) placed at the waveguide output monitored the change in light intensity directly. Signals from the photomultiplier tube were amplified (11), integrated during the flash time (12) and converted

10 by a standard 12-bit analog/digital converter (not shown) into digital format. The in-house microcomputer 12 performed fast signal averaging, and all data were adjusted for variation in flash lamp intensity by reference to a photodiode 19 placed in the monochromator. The signals were transmitted to a microcomputer 13, preferably an

15 APPLE II model, for display and storage. Two different embodiments of waveguide systems were used:

The analytical cell or cuvette illustrated on fig. 2 as one embodiment, is based on a microscope slide waveguide system. The illustrated system shows the flow cell 7 whose bottom is actually the microscope slide 8. Tightness is ensured by a gasket 20; the slides 8 were

20 placed in direct optical contact with two quarter-round silica prisms 16 and 17, preferable from Heraeus. Light was coupled into and out of the waveguide through these prisms by use of index matching oil, thereby removing the requirement for specially polished, optically

25 flat waveguide faces. The prisms were designed to allow easy adjustment of the angle of incident light θ (see fig. 1a) and to avoid contact of light with the sealing gasket 20.

The flow cell, machined from aluminium alloy, met the criterion of allowing rapid, bubble-free laminar flow along the light path.

30 Its design also ensured rapid and accurate demounting and repositioning. We chose an aluminium alloy, although other metals are also suitable, e.g. brass, because of its good thermal conductivity, relative lack of reactivity with saline solution, and low optical reflectivity after being anodized matt black to avoid stray light effects.

35 The gasket 20 was 0.5 mm thick medical grade silicone rubber and water tight under a constant sealing pressure of 2 kg/cm². Including input 18 and exit 21 ports the total cell volume was 1.8 ml, the volume

directly above the waveguide was 0.66 ml (53 x 25 x 0.5 mm) and the volume above the light path was 0.29 ml (36 x 16 x 0.5 mm).

A second embodiment (see fig. 3) is based on a fibre optic system. The fibre waveguide 31 was prepared from standard transmission optical fibres by first cutting them into 120 mm pieces, then removing the ethylene/tetrafluoroethylene coating and silicone cladding to expose an optically active surface area of 120 mm². The ends of the fibres were bared and held within specially made stainless steel end-fittings 32 and 33 (7 x 3 mm internal diameter) for support and protection. The fibre flow cell 34 was an open-ended quartz tube (internal diameter 4 mm, length 80 mm) with input 35 and output 36 tubes added for inserting and removing sample. The fibres were set in place in the flow cell with silicone rubber plugs 37, 38. Light from source 39 was filtered (40) and focussed (41) with a lens onto the end of the fibre at a mean aperture angle of 68° (see fig. 1); at the fibre output, light was refocussed by means of a lens 42 onto a photomultiplier tube 43.

The apparatus of which the essential optical components are schematized on fig. 4a comprises a dual-waveguide cell 50 of which the main walls 51 and 52 constitute two independently energized elements that transport the exciting signal originating from a source 53 and whose inner walls, either bared, blocked or coated with a specific reactant, are in contact with the analyte solution contained in cell 50. The especially shaped light conductive walls of the cuvette can be provided by usual means, e.g. by molding with a transparent plastic, for instance lucite. These walls can be made from materials with the same or different refractive indices.

The beam of light 54 originating from source 53 is alternatively split into beams 55 and 56 by a rotating chopper mirror 57a, b. On fig. 4a, this mirror 57 has been represented in two positions, i.e. one instant position corresponds to numeral 57a and another position (at about right angle to the first one) by numeral 57b. It can be readily seen that, depending on the position of the mirror 57, the beam 54 is either reflected into beam 55 or into beam 56. Thus, the light from the source 53 is alternatively injected in either portion 51 and 52 of the dual waveguide cell 50 by means of either one of a series of mirror 58a, b, c and 59a, b and c, respectively. Th

output light, respectively 60 and 61, from either part of the waveguide is then collected on a detector 62.

The remaining components of this embodiment are not represented on the drawing as being known from the art and identical to the corresponding elements disclosed in connection with the embodiment of fig. 2.

In a further embodiment, (see fig. 4b), the apparatus comprises a dual-waveguide cell 70 identical with the cell of the previous embodiment, i.e. having walls 71 and 72 acting as the two independent elements of the waveguide and operating similarly as will be seen.

The apparatus comprises a light source 73, the output of which is focussed on either side by means of lenses and mirrors, respectively 74 and 75, on the input side of guide elements 71 and 72, respectively. A chopper disk 76 with a window hole 77 acts to alternately distribute the exciting light into elements 71 and 72. The output signals from the waveguide are then directed to a detector 78 by means of mirrors 79 and 80.

In both embodiments depicted on figs 4a and 4b, one of the waveguide elements (51, 71) is coated with an antibody specific to one component to be measured in the analyte by a complexation reaction (as disclosed hereintofores) while the second element (52, 72) is left uncoated. Herein, uncoated refers to a surface without antibody. However the protein adsorption sites on this surface are usually blocked by adsorbing a protein (e.g. BSA) to the surface. Therefore during analysis, the signal collected at the output of the uncoated area reflects the interaction of the exciting beam with the bulk of the analyte, i.e. it provides the desired information on the total hemoglobin in the sample. However, simultaneously, the signal emerging from the coated side of the guide provides the required information on the component being bound by the specific reactant coated on the inner surface of this side of the cell. This will be illustrated in more detail with reference to Example 4 in this application. Suffice to say now that this kind of waveguide system (dual type) allows to gather the two types of information from separate areas of the waveguide (i.e. the phenomena are no longer superimposed like in the earlier embodiment) which may provide more accuracy in the determinations.

A variant embodiment is represented on fig. 4c. In this variant, a dual waveguide cell 90 of the same general configuration as the aforementioned cells 50 and 70 is used with the difference that ends 91a and 92a are actually made reflective, for instance by metallizing (silver) like with a mirror. Therefore, the other ends 91b and 92b, respectively, of the waveguide light conductive elements act simultaneously as input and output ends. This is illustrated by the paths of the exciting light beams provided by two sources 93 and 94 which are directed into ends 91b and 92b, respectively, after crossing beam-splitters 95 and 96, respectively. Thus, the light which penetrates through ends 91b and 92b travels through the guide first in a forward direction and then backwards after being reflected from ends 91a and 92a. This configuration enables that the interaction capacity of the exciting light with the analytes be actually doubled as compared with the previously disclosed embodiment. This variant further comprises a detector 97 for collecting the backward signals exiting from 91b and 92b and directed thereto by means of beam splitters 95 and 96 and a prism-like mirror 98. Sources 93 and 94 are alternately synchronized so that signal pulses exiting from the waveguide ends 91b and 92b do not simultaneously fall on the detector 97.

Fig. 5 is a schematic illustration at the molecular level of the phenomena taking place during analysis in a cell of the dual-waveguide type as disclosed previously. In fig. 5 areas marked 51 and 52 correspond for instance to the waveguide elements 51 and 52 depicted on fig. 4a. The area intermediate between areas 51 and 52 represents schematically an analyte medium with species dissolved therein and reactants or species attached to the inside walls of elements 51 and 52. Element 51 is pictured to have deposited thereon antibodies 100 specific to HbA_{1c} entities labelled 101. Some of these HbA_{1c} molecules are shown after complexation with the specific antibody 100, others are still free. The other surface (i.e. the surface of element 52) is shown coated with blocking agents 102 (for instance, bovine serum albumin) said agents being intended to minimize the possible affinity of the bare wall to other species in solution, for instance HbA_0 103 and other proteins of any type 104.

Thus, during analysis, non specific binding of Hb to surface

52 is prevented (or at least strongly minimized) which makes it possible to measure the bulk hemoglobin by the interaction of the evanescent wave component of the signal travelling in 52 with the analyte solution at depths beyond that of the blocking coating deposited on the surface.

In contrast, a complexation reaction occurs on surface 51 between the antibody molecules 100 coated thereon and the $\text{HbA}_{1\text{C}}$ (AG) molecules in the analyte solution. This reaction although rapid is not instantaneous; therefore a layer of complex progressively builds up on surface 51 with consecutive corresponding interaction with the light component travelling in that element of the waveguide, this resulting in the production of response curves of the A or B type depicted in fig. 6 (see the Examples that follow).

In order to practically carry out the tests, the microscope slides were cleaned by consecutive immersion in concentrated sulfuric acid and distilled water, ethanol, and acetone, using standard slide staining glassware. Fibres were cleaned in ethanol ultrasonically and, supported on glass rods, were immersed in the various antibody solutions. Antibodies were either physically adsorbed to the surface of the waveguides or covalently coupled. Adsorption was carried out by incubating cleaned waveguides with solutions of antibody (5 mg of protein/ml of 0.05 mol/l Tris Hcc buffer, pH 7.0) for four hours. Unadsorbed proteins were washed away with saline and residual protein binding sites blocked by incubation of the antibody-coated waveguides with bovine serum albumin (1.0% by weight in TRIS Buffer). The method of coupling was essentially that of Weetall, involving aminopropyltriethoxysilane APTS (Immobilized Biochemicals and Affinity Chromatography, R.B. Dunlop, Plenum Press, New York, p. 191-212) in an acid aqueous silanization environment. (Immobilized Enzymes, Antigens, Antibodies and Peptides: Preparation and Chromatography, 1: Enzymology, H.A. Weetall, Marcel Dekker Inc. New York 1975, p. 1-48).

In general, we reacted waveguides with APTS (0.4 mol/l) for 3 hours at 80°C. We then heated the slides or the cuvette walls at 120°C and the fibres at 100°C for 2 hours, then let them soak in glutaraldehyde solution (0.5 mol/l) in phosphate buffer (0.1 mol/l, pH 6.8) for 90 min at ambient temperature. The "activated" waveguides were

then reacted with antiserum Ab (5 mg of protein per milliliter of phosphate buffer) for 24 hours at 4°C. After washing the antibody-coupled waveguides in phosphate buffer, we stored them at 4°C in isotonic saline (0.14 mol/l, containing sodium azide, 8 mmol/L). Measurements of protein (Anal. Biochem 51, 654-655 (1973)) before and after the coupling demonstrated protein uptakes of approximately 1 µg/cm² of quartz.

The following Examples illustrate the invention in more detail.

10

Example 1 (to demonstrate the existence of two distinct optical phenomena: i) interaction of the evanescent wave component with hemoglobin in bulk,
ii) interaction of the evanescent wave component with the Ag/Ab complex under formation).

15

The apparatus used was that of embodiment of fig. 2.

Preparation of standards with known solutions of hemoglobin.

Purified hemoglobin A (HbA) was obtained from SERVA FEINBIOCHEMICA, Heidelberg, FRG. Bovine Serum Albumin (BSA) was from SIGMA CHEMICAL CO., ST. Louis, MO, USA. All chemicals for buffers and solvents were Analar or Reagent Grade from MERCK, Darmstadt, FRG, or BDH, Poole, Dorset, UK. Rabbit antiserum to human HbA was purchased from DAKO, Copenhagen, Denmark.

25 Waveguides were fused-silica microscope slides (Suprasil 1.75 mm x 25 mm x 1 mm) from HERAEUS QUARZSCHMELZE GmbH, Hanau, FRG.

Slides were cleaned by consecutive immersion (10 min each) in concentrated sulphuric acid, distilled water, ethanol and acetone. Antibody was coated to the surface by incubating the cleaned slides for 1 hour in a solution of anti-HbA diluted five-fold in phosphate buffered saline (PBS; 0.1 mol/L phosphate, pH 7.4, 0.9% (w/v) NaCl). Following rinsing with distilled water, remaining protein binding sites were blocked by incubation for 1 hour with 1% (w/v) BSA in PBS. Slides were then rinsed in distilled water and stored at 4°C in isotonic saline prior to use.

35

Slides were fixed in conformity to the first embodiment illustrated on fig. 2 in a manner which allowed light to be coupled in-

to the slides at different angles θ . The flow cell 7 was fixed to the surface via a 0.5 mm silastic gasket 20 and bubbles purged from the system by pumping assay buffer (PBS + 5.0% (w/v) BSA) through the cell. Standard Hb solutions (1.0, 0.5, 0.1, 0.05 mg/ml) were made up in assay buffer to give a final protein concentration of 5 mg total protein/ml.

The assay procedure was initiated by injecting 3.5 ml of standard Hb solution into the cell after establishing a base-line signal. The wavelength of the input beam was selected by adjusting the monochromator at 410 nm and the reaction was monitored by the reduction in intensity at 410 nm. The angle θ was first selected at random above 66° (the critical angle). A value of about 67° was used in the test reported below.

Illustrated by fig. 6 are the antibody-binding curves obtained with the 1.0 (curve A) and 0.1 mg/ml (curve B) Hb standards, using successive antibody-coated slides. After stabilising the base-line, the standards were injected at t_0 and an immediate fall (I_A , I_B) in transmission (arbitrary units) was followed by a slower but still rapid binding event which continued over the next 10 minutes. The initial fall was due to free hemoglobin molecules optically absorbing within the D_p range of the evanescent wave (see fig. 1). Note that at this early stage the complex layer is starting to form; therefore the evanescent wave component extends quite significantly beyond the initial Ab coating and is free to interact with the bulk solution. The subsequent slower change in signal of rate K and magnitude, respectively, M_A and M_B at time t_1 was due to antibody-binding of Hb at the surface. In the absence of antibody the signal changed minimally after the initial immediate fall. This was shown in a control experiment not shown on the drawing using no Ab coating.

Then the cell was washed with assay buffer (t_1) which removed all unbound materials. The residual absolute change in signal (WA , WB) is related to dose as indicated in the table below.

<u>Absolute change in signal</u>			
	<u>Test</u>	<u>Replicate</u>	<u>Mean</u>
Dose	1	2	
5 Hb			
(mg/ml)			
0.1	- 4.6%	- 4.3%	- 4.3%
10 1.0	- 14.9%	- 12.5%	- 13.7%

The standard curves A and B were usable as templates for the determination of hemoglobin in unknown samples of blood. Similarly reproducible information could be gathered from measured values M_A and M_B provided unknown samples were measured after a constant time t_1 .

Example 2

20 Optimalization of the incident angle (θ) vs sensitivity.

With an antibody-coated slide in place, Hb standard solution (1 mg/ml) was injected into the flow cell. After 10 minutes reaction the unbound materials were washed out of the cell with assay
25 buffer. Bound materials were monitored by the reduction in transmission at 410 nm. The effect of incident angle of light was investigated by varying the angle (θ) from 64° to 78°. The critical angle (θ_c) is 66°. The results were plotted as the % transmission decrease (= sensitivity) vs angle of incidence (fig. 7). It can be seen that
30 with this system measurement of antibody-binding of Hb is possible with angles between 66 at 70°, with an optimum near to 68°. Larger angles have too small a penetration depth in this case although they may suit analytical system of a different kind; smaller angles result in refraction not reflection. Angles between 66 and 68° are less suitable
35 presumably because the depth of penetration of the evanescent wave component is too great.

Example 3

Preparation of more refined standard curves.

5 Hb standard solutions were incubated with separate antibody-coated slides and the reaction monitored using θ = about 68° as the optimal angle. The results expressed as % transmission, show a dose-response relationship:

10	<u>Standard Hb</u> (mg/ml)	<u>% Transmission</u>
	1.0	87.2
	0.5	94.0
15	0.1	94.4
	0.05	95.3
	0	100

The minimum detection limit of this system is about 0.1 mg/ml or 0.1 g/l. Normal adult HbA values are 135 - 175 g/l, normal HbA1c levels are 4 - 9 g/l, thus this method can be used with $\times 10$ - $\times 100$ dilution of normal samples with adequate sensitivity.

Example 4

25

Measurement of hemoglobin in the presence of foreign hemoglobin.

Solution samples were prepared based on avian hemoglobin (pigeon) and containing variable proportions of human hemoglobin to be measured. The total of both hemoglobins was always 5 mg/ml and the proportions of human hemoglobin are given in the Table below. A dual waveguide of the type shown in figs 4a and 4b was used, one of the surfaces (e.g. 51) being coated with antibody to human IgG. The other surface (52) was blocked with bovine serum albumin as usual.

35

Upon doing the measurements a sharp dip (I) corresponding to 75.3% transmission was observed in all cases; then the further drop in transmission (M) (cf. with Example 1 and fig. 6) was recorded after

an interval of 10 min. In the case of the sample containing only avian hemoglobin, no further change during the 10 min interval was observed. The results are summarized below.

5	Human Hb in	Transmission (%)	M
	avian Hb (%)	(after 10 min)	
	0	75.3	0
	1	74.9	0.4
10	2	74.4	0.9
	10	72.0	3.3
	20	68.3	7.0

Thus the value recorded for the first initial dip I can be correlated with the total hemoglobin present while the values (M) observed after the 10 min reaction period and corresponding to the binding of the human hemoglobin factor to the antibody coated on surface 51 can be correlated with the human hemoglobin content of the sample and its ratio to total hemoglobin. Standard curves were made from the above data by recording on an automatic recorder coupled to the apparatus used in this Example. Such curves were thereafter used as comparative data for determining unknown mixtures of human hemoglobin in avian hemoglobin.

25 Example 5

Measurement of glycosylated Hemoglobin ($\text{HbA}_{1\text{C}}$) in the presence of hemoglobin.

30 Standard glycosylated Hb ($\text{HbA}_{1\text{C}}$) was prepared from pooled heparinized whole blood by cation-exchange chromatography (L.A. TRIVELLI et al., New England J. of Medicine 284 (1971), 353), using Bio-REX 70 resin (BIO-RAD, Richmond, Ca, USA). The purified $\text{HbA}_{1\text{C}}$ was then used to prepare standard samples by recombining it in varying known amounts with blood free from the glycosylated hemoglobin. The concentrations of $\text{HbA}_{1\text{C}}$ relative to total hemoglobin in the samples varied from 1 to 20% by weight and the total Hb concentration was

of the order of 150 g/l.

An analytical apparatus with cuvettes involving a dual waveguide as illustrated on fig. 4b was used for the determinations; the inner surface of one side of the cuvette was plated with antibody specific to HbA_{1C} while the surface of the opposite side was left free. The content of each cell (a fresh one was used for successively testing each standard) was about 1 ml and 0.1 ml of the standard to be measured with about 0.9 ml of PBS were pipetted therein. Fig. 8 depicts one of the titration curves obtained after 15 min incubation time (with the 20% HbA_{1C} sample), the upper curve (nearly flat) being that recorded with the uncoated part of the guide and the lower curve showing the response of the antibody coated part of the waveguide.

The results of the analysis of the various standards are also gathered in the table below.

	Standard sol. under test (% HbA _{1C})	(% Transmission in waveguide)		Difference M (%)
		uncoated side	coated side	
20	0	56.1	55.8	0.3
	1	55.5	54.7	0.8
	5	55.7	50.2	5.5
25	10	58.0	49.2	8.8
	20	54.9	42.4	12.5

The difference of 0.3% for the zero HbA_{1C} sample may indicate some degree of residual affinity of the HbA_{1C} specific antibody for the deglycosylated blood medium. This factor is however considered negligible under practical analytical conditions.

It should also be noted that the % transmission in the uncoated part of the waveguide was not constant from one cell to the other seeming to indicate that the method is not suitable for accurately determining total Hb. However it is not necessary in this instance to measure total Hb, but only to relate the signals from the uncoated and coated sides. Secondly, it is difficult to maintain a degree

of constancy in manually fabricating a series of cuvettes such that each will enable full reproducibility of absolute measurements without initial calibration of the equipment. Undoubtedly, when cuvettes are manufactured industrially by molding on a large scale, this
5 disadvantage is overcome.

Example 6

The simultaneous determination of human IgG and human serum albumin (HSA) by fluorescence type assay.
10 min

The dual waveguide system as in the previous Example was used, a cut-off filter to block the incident radiation at 490 nm and pass the fluorescent signal at 520 nm being inserted on the light path
15 before detector 78. The exciting light was generated by a monochromator of the type (9) disclosed in connection with fig. 2.

One wall surface (A) of the cuvette serving as the dual-waveguide was coated with sheep raised antiserum against IgG. This was effected by adsorption according to usual means using a diluted solution of antiserum (γ -chain specific; SAPU, Carlisle, Scotland; dilution by volume 1/400). The oppositely facing wall (B) of the cuvette was coated by the same technique with sheep antiserum against HSA (final dilution 1/100 by volume) obtained from the same source.
20

Then, mixed combination standard solutions were prepared by dissolving together human IgG (SERVA BIOCHEMICALS) and HSA (UCB-BIOPRODUCTS, Brussels, Belgium). Concentrations of reagents in the standards were 0.1; 1.0; 10.0 and 100.0 $\mu\text{g/ml}$. The solution buffer used as the standard medium was phosphate buffer 0.05 mole/l (pH 7.4); 0.9% NaCl (W/v); 0.05% NaN_3 (W/v); Tween 20 (SIGMA) 0.1% (v/v) and
25 30 2% (v/v) normal sheep serum (SAPU).

The test disclosed in this experiment was based on making a "sandwich" type assay i.e. the cuvettes were contacted with the standards and incubation was carried out for a determined period to ensure sufficient binding of the antigens on the respective specific antibodies attached to surfaces (A) and (B). This incubation time was an
35 accurately measured 10 min period in the course of which the amount of overall bound antigen was in proportion to its concentration in

the standard. Tests against blanks (zero % of antigen reagents) were run identically.

Thereafter, the cells were emptied and rinsed of all unbound materials and a combined solution of second antibodies to the anti-
5 gents attached to the waveguide surface was added. This combination solution contained 1/40 (v/v) buffer diluted rabbit anti-HSA and rabbit anti-IgG (obtained from DAKO IMMUNOGLOBULINS) labelled with fluorescein (fluorescein isothiocyanate, FITC was used as the actual marker according to usual means).

10 One the fluorescent labelled mixed antibody solution was added, an instantaneous fluorescence rise was observed at the output of the waveguide followed by a slower rate dependent signal (see fig. 9) whose height after a given period was in proportion to the standard concentrations in IgG and HSA taken independently. After decoding,
15 the signals components originating from surfaces (A) and (B) were displayed separately and the results are gathered in the following table.

Concentration 20 of IgG and HSA in standards (ng/ml)	Response from surface A (arbitrary units)			Response from surface B (arbitrary units)		
	Test	repeat	average	Test	repeat	average
0.1	1	2	1.5	-1	2	1
1.0	27	24	25	44	44	44
25 10.0	64	61	62	60	56	58
100.0	160	170	165	140	135	137

Fig. 9 shows graphically for components A and B the situation from zero to 15 min in the case of the 1 µg/l (dashed line) and 10
30 µg/l (mixed line) standards. The full line represents blanks.

As in the previous Example, samples with unknown concentrations of IgG and HSA were run identically and ascertained by comparing with the standards.

C L A I M S

1. A method for ascertaining parameters in a liquid analyte, e.g. determining species in solution, in which the evanescent component of a fully reflected light signal interacts, at the interface between said analyte and a waveguide of said signal, with a monomolecular layer of a complex constituted of one of said species and a reagent specific thereto attached to the guide surface and/or the bulk of the analyte, thus providing a light energy output representative of said parameters, the method including collecting said output energy and processing it opto-electronically into readout data corresponding to said parameters, characterized in either:

a) controlling the angle of reflection of said light signal in the waveguide for allowing said evanescent wave component to penetrate beyond said complex layer into the bulk of the solution to a depth sufficient to be responsive to both said complex layer and said bulk of the analyte and simultaneously provide processable output energy on a parameter representative of a first species in said complex layer and a parameter representative of a second species in the solution, or

b) using two or more distinct areas of the waveguide, one first area retaining a first reagent attached thereto to provide a complex layer of a first species to be determined, whereby said interaction is only responsive to said complex layer and the output is only representative of a parameter of this first species, and a second area, the latter being either:

(b1) bare or blocked, whereby said interaction is responsive to the bulk of the analyte and representative of a parameter of a second species dissolved therein or

(b2) coated with a second reagent specific of a second species thus providing a layer of a second complex to which said interaction is responsive and the output is representative of another parameter, the latter pertaining to said second species,

c) processing the output consisting of a mixed signal and splitting it into components specific of each parameter of interest,

d) expressing said components into separate data, and

e) ascertaining said parameters by correlating the displayed data with standard data obtained from standard solutions in which the corresponding parameters are known.

2. The method of claim 1, wherein the excitation light in the
5 waveguide is transmitted by multiple reflections therein and wherein the interaction of this signal with the analyte involves some degree of absorption, and/or scattering, and/or the generation of fluorescence.

3. The method of claim 2, wherein the waveguide consisting of
10 an optical slide or fiber, a layer of complex forms during analysis at the surface thereof thus providing a time dependent response on said first species and instant response on said second species.

4. The method of claim 2, wherein said two or more distinct areas of the waveguide are energized by the same incident light signal.

15 5. The method of claim 2, wherein said two or more distinct areas of the waveguide each involve separately illuminated waveguide elements.

6. The method of claim 5, wherein said waveguide is constituted by an analytical cuvette whose oppositely facing walls operate as
20 independent separately illuminated elements.

7. The method of claims 3 to 5 wherein said mixed signal collected at the output comprises components at the same or different wavelengths.

8. The method of claim 5, wherein the illumination of said dis-
25 tinct areas is done in alternation.

9. The method of claim 1 for substantially simultaneously determining total hemoglobin (Hb) in a blood sample and, selectively at will, further hemoglobin factors or derivatives thereof such as glycosylated hemoglobins (Ag) relative to said total hemoglobin, this
30 method comprising the following steps:

a) Coating at least part of the surface of an optical waveguide system of a refractive index (n_1) greater than that (n_2) of the sample to be analyzed with one or more coatings of complexing reactants (Ab) each coating being of a reactant specific to that factor or deriva-
35 tive of hemoglobin Ag and capable of building up a layer of said complex upon reaction with said Ag;

b) Illuminating said waveguide at an input end with a beam of

light and collecting the exiting light at an output end, said beam being transmitted along said guide by a multiple internal reflexion mechanism at an angle θ such that the effective range of action outside the guide of an evanescent light component associated with said beam transmission exceeds that of said complex layer;

c) Contacting together said blood sample to be analyzed and said illuminated waveguide whereby, on one hand, part of the light travelling in the guide is initially absorbed by interaction of the evanescent wave component with the hemoglobin of the bulk sample, this resulting in an instantaneous sharp dip (I) in the exiting light from said output end and, on the second hand, an immuno-type reaction develops between said glycosylated hemoglobin or other factor to be determined in said blood sample and corresponding Ab coated on the guide with consecutive build-up of said complex layer, such development resulting in a relatively slower change to said exiting light, said relatively slower change being due to the interaction of said evanescent wave component with said complex layer in formation;

d) Observing, measuring and/or recording said sudden optical absorption dip (I) occurring to the transmitted light collected from said output end, said measured dip being quantitatively related with the concentration of the total hemoglobin Hb of the sample;

e) Observing, measuring and/or recording said relatively slower change, the magnitude (M) and rate (K) of which quantitatively relates to the amount of glycosylated hemoglobin or other factor Ag in the sample;

f) Effecting the required computations e.g. electronically, to express the results obtained from the values of I and M or K in terms of concentration of Hb and/or ratio of Ag to Hb in the sample.

10. The method of claim 9, wherein steps (d) and (e) are both effected on the signal resulting from the interaction of the incident light and the analyte at the area of the waveguide bearing the antibody Ab coated thereon.

11. The method of claim 9, wherein step (d) is effected on the component of the output signal resulting from said interaction on a part of the waveguide not coated with the antibody Ab.

12. The method of claim 11, wherein there is used a dual type

waveguide, one portion of which is coated with antibody Ab and another portion of which is not coated with antibody.

13. The method of claim 12, wherein said other portion is coated with a blocking protein for minimizing the possible deposition of
5 hemoglobin on the waveguide uncoated surface area.

14. The method of claim 12, which comprises using as a dual waveguide an analytical cuvette whose main opposite walls are light conductive and made of a transparent material of suitable refractive index n_1 greater than that (n_2) of the blood sample, this material
10 serving as the waveguide.

15. An apparatus for the combined determination by optical means of total hemoglobin together with at least one further specific hemoglobin factor in a sample of blood using a waveguide carrying a light signal capable of interacting with said species to be determined and
15 providing detectable responses representative therefrom, said interaction being that of the evanescent wave component of the light signal travelling in the waveguide with either bulk of the sample or a complex resulting from the binding of said factor by a reactant specific thereto attached by coating on the waveguide, this apparatus in-
20 cluding

a) a light source means for generating a light beam and injecting it into a suitable input end of said waveguide;

b) a light detecting means for collecting the light signal exiting from an output of said waveguide and generating an electric signal
25 representative of said interaction;

c) a computing means for processing said electric signal and providing suitable readouts on said desired determination,

characterized in that said specific reactant coating involves only part of the operative area of the waveguide, the remainder thereof being either bare or rendered inhibitory to the binding of said
30 factor by means of a blocking agent and in that said reactant coated area of the waveguide is responsive to said specific factor to be determined, whereas said uncoated or blocked area is responsive to said total hemoglobin.

35 16. The apparatus of claim 15, wherein the wave guide has a dual-type structure and comprises two independent optical elements to simultaneously or alternatively carry said optical signal, one of said

elements being reactant coated and the other being bare or blocked.

17. The apparatus of claim 16, using as the waveguide an optical cell or cuvette of which two oppositely facing walls operate as said waveguide elements.

5 18. The apparatus of claim 17, in which said light source means comprise chopper means to alternatively inject the signal light into the two elements of the dual-waveguide.

19. The apparatus of claim 18, in which said chopper means are either a rotating mirror or a chopper disk.

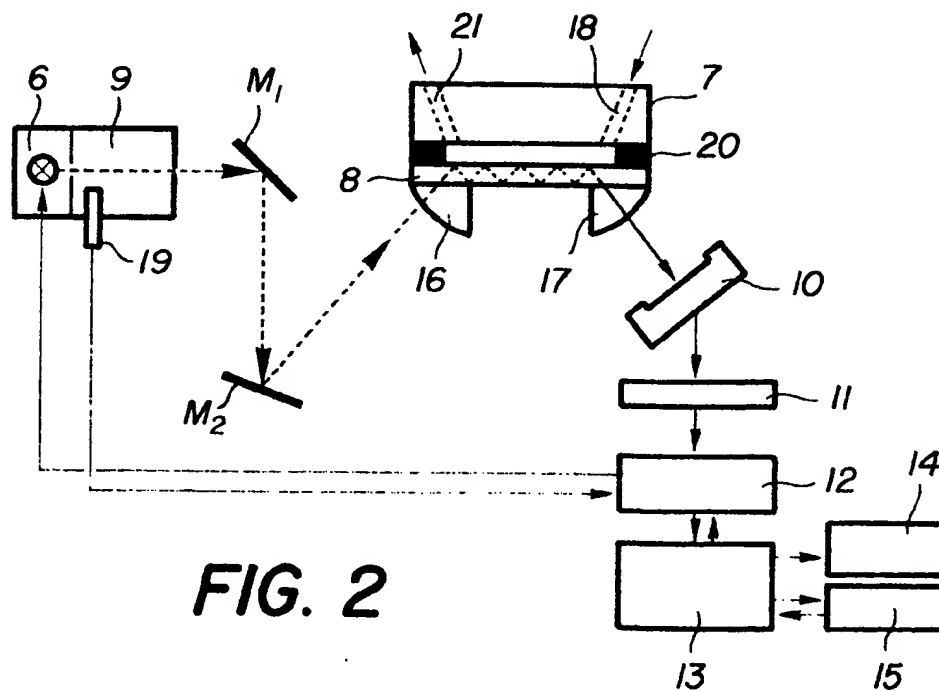
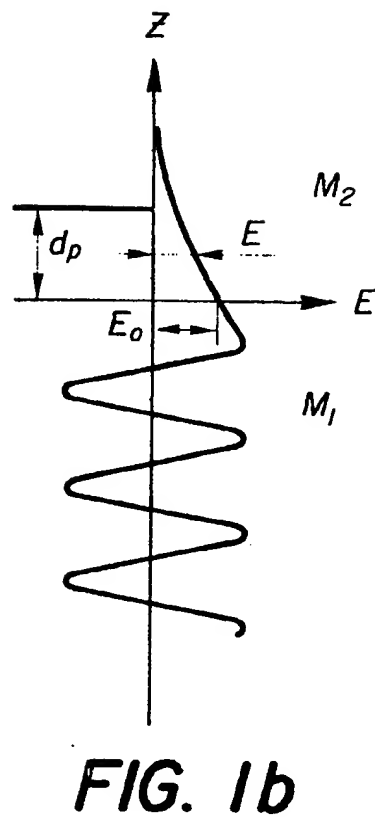
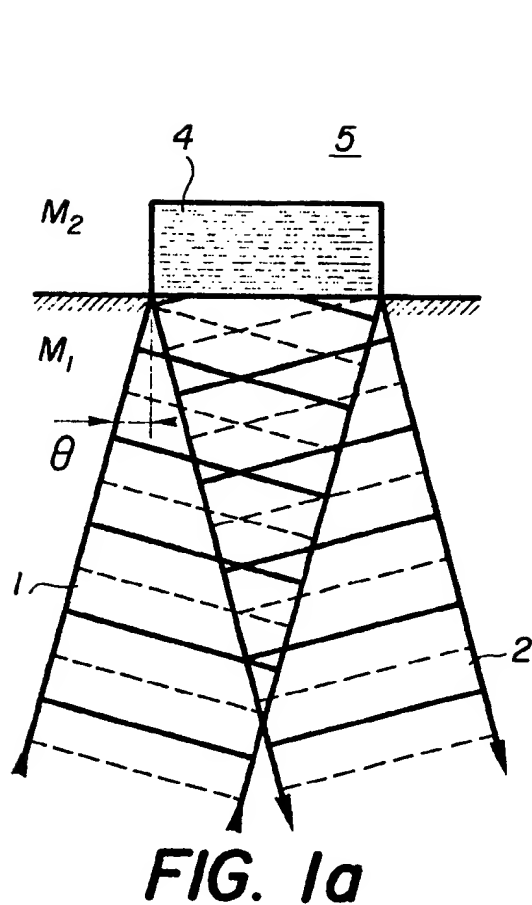
10 20. The apparatus of claim 16, in which said light source means comprise two independent alternately flashing light sources the output of which is focussed each on one optical end of said elements via beam splitting means and in which the other end of said elements is made totally reflective so that the light signal carried by said
15 elements travels forwards and backwards therein.

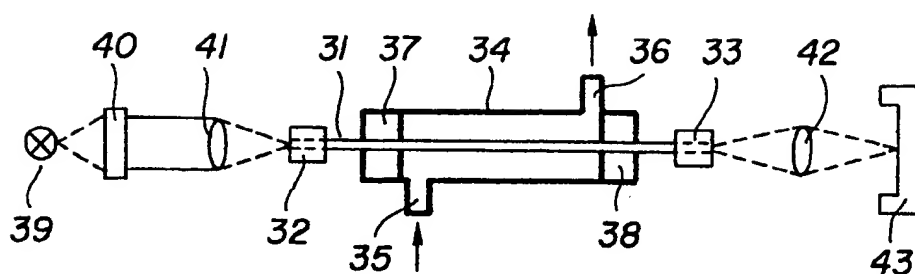
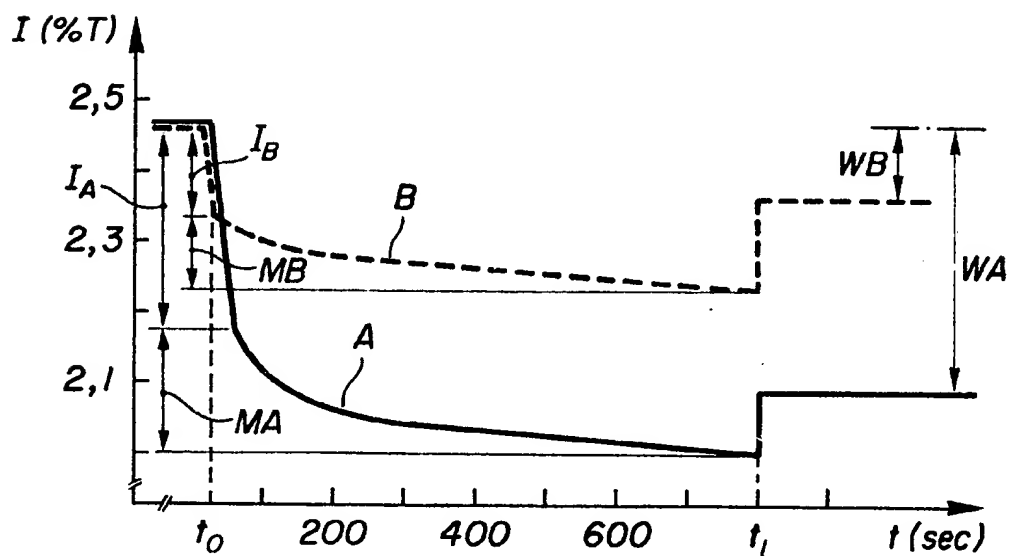
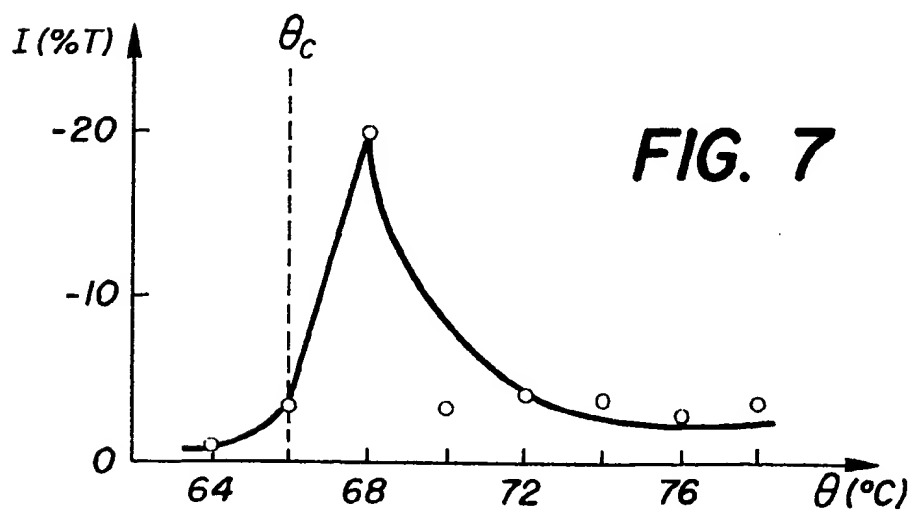
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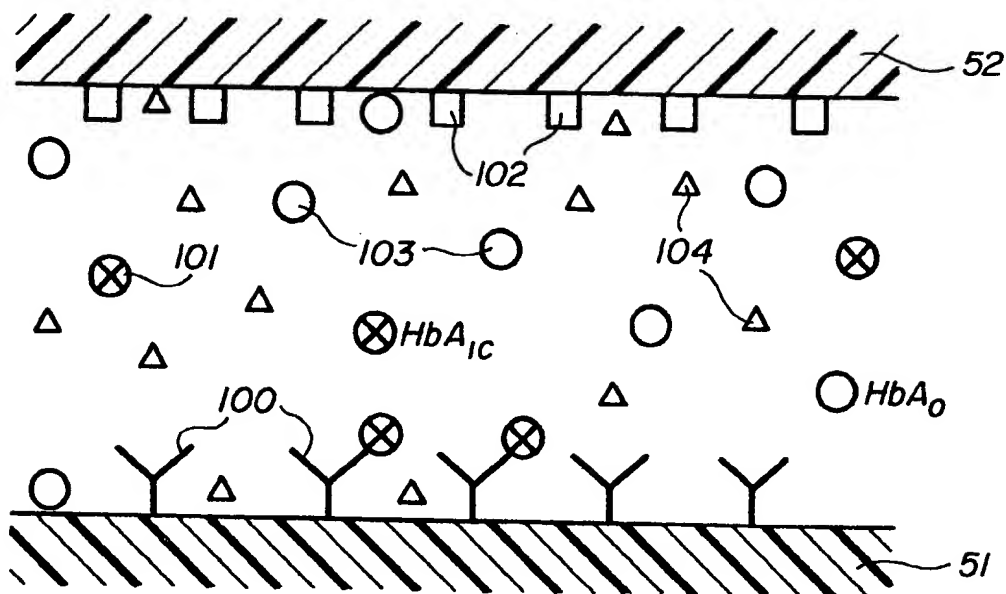
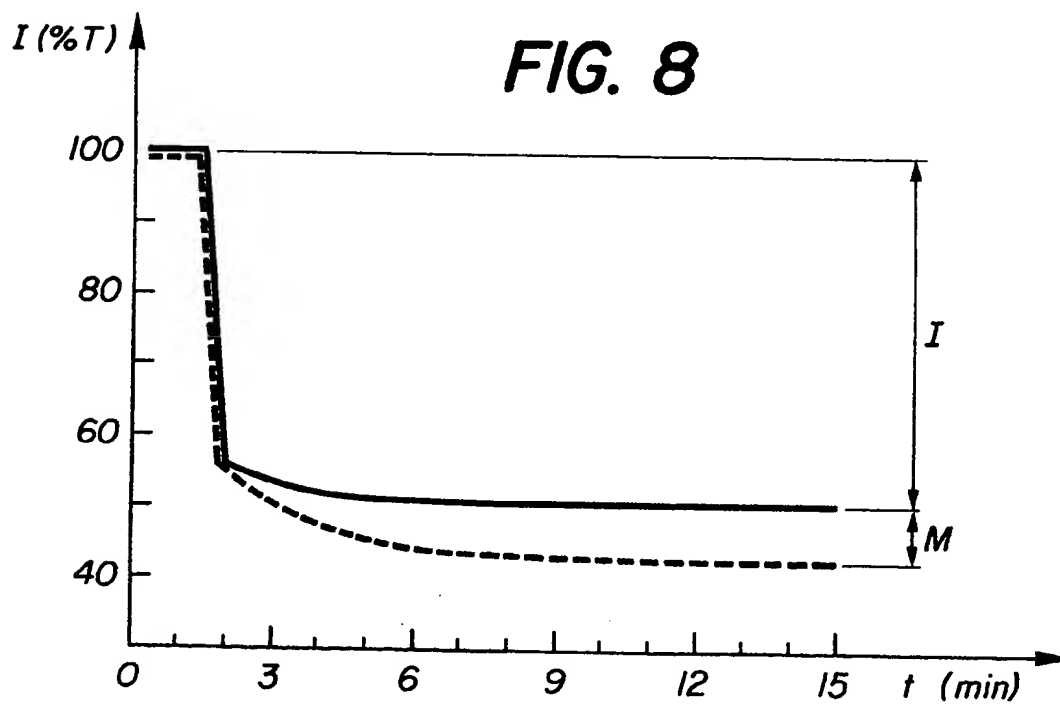
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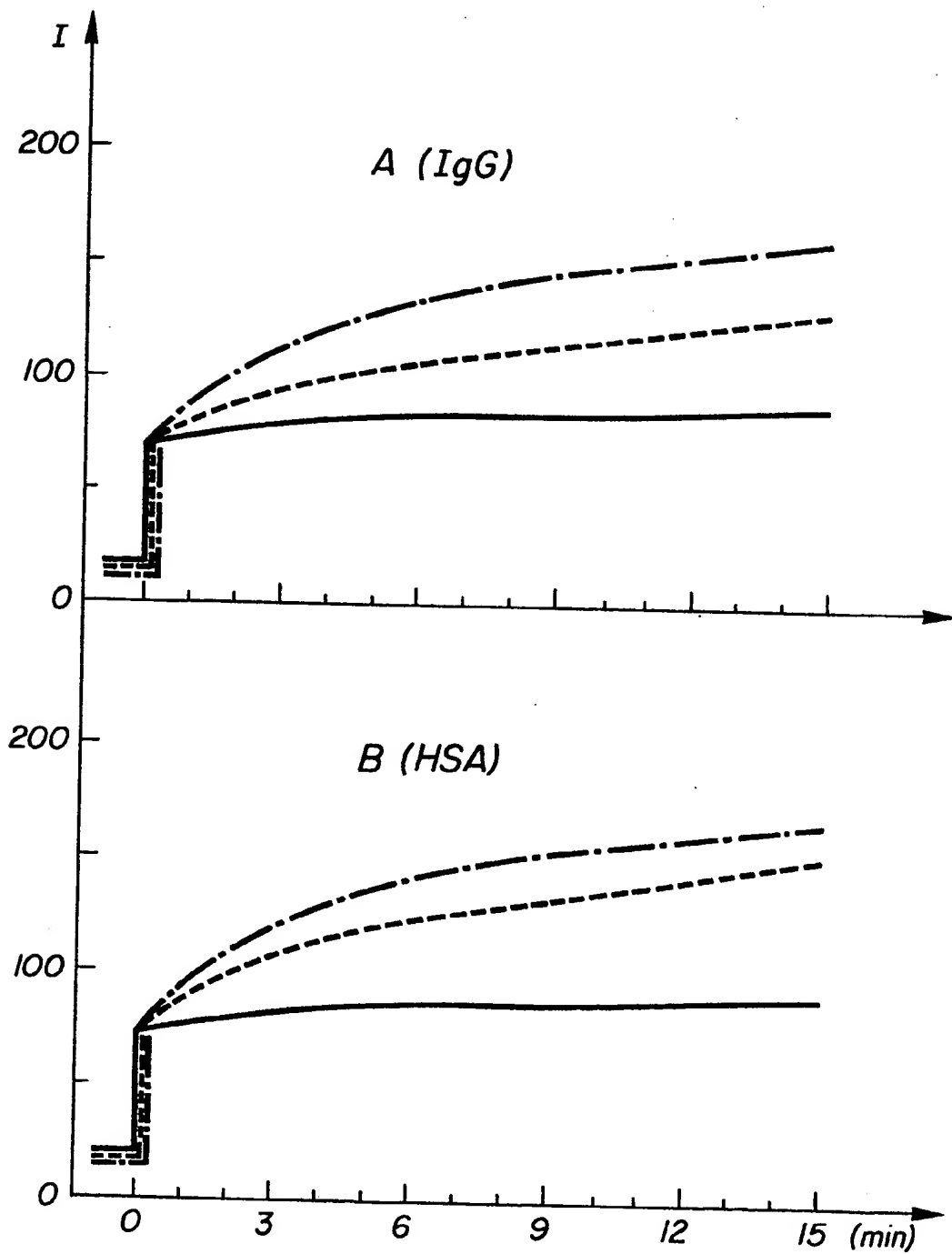
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**FIG. 3****FIG. 6****FIG. 7**



**FIG. 5****FIG. 8**

**FIG. 9**



European Patent
Office

EUROPEAN SEARCH REPORT

0184600

Application number

EP 84 81 0600

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A,D	EP-A-0 075 353 (BATTELLE MEMORIAL INT.) * Figures 1,4; figures 9,10 *	1	G 01 N 21/75 G 01 N 33/552 G 01 N 33/72
A,D	US-A-3 939 350 (M. KRONICK et al.) * Columns 2,4,6 *	1	
A	US-A-4 169 676 (N. KAISER) * Column 4; figure 3 *	1,5-8, 18-19	
A	US-A-4 255 053 (D. LÜBBERS et al.) * Column 2; figure 2 *	1	
P,A	EP-A-0 121 442 (SYVA CO.) * Pages 2-4,7 *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) G 01 N 21/77 G 01 N 21/55 G 01 N 21/64 G 01 N 33/552 G 01 N 33/72 G 01 N 21/75
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 16-08-1985	Examiner BOEHM CH.E.D.
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